Response of heterogeneous ribonuclear proteins (hnRNP) to ionizing radiation and their involvement in DNA damage repair

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Running Head: Role of hnRNP proteins in Radiation Response

Response of heterogeneous ribonuclear proteins (hnRNP) to ionizing radiation and their involvement in DNA damage repair

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Acronyms:

ATM, Ataxia Telangiectasia Mutated kinase

ATR, Ataxia Telangiectasia Rad3 related kinase

CHiP, Chromatin immunoprecipitation

CIRP, cold-inducible RNA-binding protein

Cs-137, cesium-137

Co-60, cobalt-60

DDR, DNA damage response

DSB, double-strand breaks

DNA-PK, DNA-dependent protein kinase

Gy, gray

GSK3β, glycogen synthase kinase 3 beta

HDM2, human homologue of murine double minute

hnRNP, Heterogeneous ribonucleoprotein

HR, homologous recombination

IR, ionizing radiation

Ku, Ku antigen, 70kDa and/or 80 kDa subunit

MDM2, murine double minute, a p53 ubiquin ligase (mouse)

NGF, Nerve Growth Factor

NHEJ, non-homologous end joining

p21, Cyclin-dependent kinase inhibitor 1A

p53, tumor protein 53

PIKK, phosphatidylinositol 3-kinase-like kinase

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RNP, ribonuclear proteins

RPA, Replication protein A

TERT, Telomerase Reverse Transcriptase

TLS/FUS

hnRNP P2

UV, ultraviolet

UP1, Unwinding Protein 1

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Abstract

Purpose: To determine the relationship between heterogeneous nuclear ribonucleoproteins (hnRNP) and DNA repair, particularly in response to ionizing radiation (IR).

Materials and Methods: The literature was examined for papers related to the topics of hnRNP, ionizing radiation and DNA repair.

Results: Sixteen different hnRNP proteins have been reported to show either mRNA transcript or protein quantity changes following ionizing irradiation. These protein modifications of hnRNP in response to ionizing radiation have also been noted; hnRNP A18, C1/C2 and K are phosphorylated; hnRNP C1/C2 is among the early subjects of radiation induced apoptotic proteases; and hnRNP K degradation is under the control of murine double minute ubiquitin ligase (MDM2). HnRNP orchestrate the processing of bound mRNA in response to different stresses including irradiation and interact with important proteins from DNA Damage Response (DDR) pathways including DNA-dependent protein kinase (DNA-PK), the Ku antigen (Ku) and tumor suppressor protein 53 (p53) which bind hnRNP A18, B1, C1/C2 and K respectively. Irregularities in the expression of hnRNP A18, B1, K, P2 and L have been linked to cancer and radiosensitivity. The accumulated evidence points to a role for hnRNP A1, A18, A2/B1, C1/C2, K and P2 in regulating double-stranded break repair pathways by promoting either homologous recombination (HR) or non-homologous end rejoining (NHEJ) repair pathways following ionizing irradiation.

Conclusions: HnRNP proteins are among the critical factors coordinating repair pathways following exposure to ionizing irradiation, through both protein-protein

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interactions and control of transcription and translation of key repair and stress response mRNA.

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Introduction

In eukaryotic cells pre-mRNA molecules are packaged, processed, exported and localized to production sites in the cytoplasm by a dynamic complex of diverse proteins known as ribonuclear proteins (RNP). These proteins follow RNA from transcription in the nucleus to translation in the cytoplasm (Krecic and Swanson, 1999, Dreyfuss et al., 2002). RNP are involved in gene regulation through a plethora of protein-protein, protein-RNA and protein-DNA interactions that connect them to the major regulatory networks of the cell.

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Most of the human genome is represented by primary transcripts, including non-coding portions (Weinstock, 2007). Only 2-3% of this transcriptional output is RNA that codes for protein, while the non-coding majority is spliced from pre-mRNA and sent to the exosome for processing (Mattick, 2003). Additionally, the majority of eukaryotic mRNA expresses distinct localization patterns throughout the cell (e.g. 71% of 3370 genes analyzed by florescent *in situ* hybridization assays in embryonic *Drosophila* cells showed clear distribution patterns)(Lécuyer et al., 2007). These critical cellular regulatory functions, transcript processing and localization are regulated by RNP complexes which escort pre-mRNA to the cytoplasm. The overwhelming diversity of these proteins, their multitude of structural conformations, binding specificities and isoforms implies that RNA transport and processing are highly specialized functions (Krecic and Swanson, 1999, Dreyfuss et al., 2002).

Heterogeneous nuclear ribonucleoproteins (hnRNP or sometimes hnrp) make up a significant subclass of known RNP. They are defined by three attributes: 1. They can be co-immunoprecipitated. 2. They show RNA binding capacity. 3. They do not belong to other defined classes of RNP such as small nuclear RNP (Dreyfuss et al., 1993, Shyu and Wilkinson, 2000). HnRNP were first described as a group of 6 chromatin associated RNA binding proteins which bound nascent polymerase II transcripts (Dreyfuss et al., 1993). Subsequent co-immunopurification, gene cloning and sequence analysis revealed a group of 30 abundant proteins and other less common hnRNP isoforms (Dreyfuss et al., 2002). The number of known hnRNP continues to grow and sometimes well studied proteins are later identified as hnRNP. For this reason, nomenclature inconsistencies exist in the literature. This review will defer to the 'hnRNP' designation and make note of alternative names where relevant.

HnRNP partially compose, bind or unbind to an evolving and unique complex of RNP and RNA which determines the fate of transcripts (Dreyfuss et al., 2002). They are well tied to the control networks of the cell through interactions with DNA and proteins. HnRNP do not necessarily exhibit homology to one another, but there is, very often, substantial overlap between their structures and functions.

This document will focus on the role of specific hnRNP in the cellular response to ionizing radiation (IR). IR plays a role in both the induction and the treatment of cancers. Within the cell, exposure to IR may cause direct and indirect DNA damage, genotoxic stress, general transcription arrest and necessitate the initiation of repair pathways (Ward, 1988). To begin the DNA damage response (DDR) pathway six phosphatidylinositol 3-kinase-like kinases (PIKK) are activated, including ataxia telangiectasia mutated (ATM)

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protein, ATM and rad3 related (ATR) protein, ATM nerve growth factor (NGF), and DNA-dependent protein kinase (DNA-PK). Activated, these proteins phosphorylate downstream target proteins which coordinate the process of DNA repair (Abraham, 2004).

Double stranded breaks (DSB) in genomic DNA are considered to be the most deleterious form of DNA damage caused by IR. DSB may be repaired through either the homologous recombination (HR) pathway regulated in part by Recombination protein A homologues (hRad51, hRad52 and hRad54) or through the non-homologous end joining (NHEJ) pathway regulated in part by DNA-PK, and Ku antigen (Ku) (see (Sonoda et al., 2006, Weinstock et al., 2006) for review). With respect to aiding cell survival and introducing mutations into genome, both of these repair pathways have their own peculiar strengths and weaknesses. While HR has the potential to restore the complete sequence information for damaged genes, too much HR may lead to a loss of genomic heterogeneity, gross chromosomal rearrangements and ultimately tumorgenesis (Henning and Stürzbecher, 2003); also, HR is possible only during late S and G2 phases of the cell cycle after the cellular DNA has been replicated. On the other hand, NHEJ can occur at any point of the cell cycle but it is more error prone than HR and therefore less likely to restore the functionality of damaged sequences at the site of DSB; nevertheless, NHEJ generally prevents mitotic death and severe structural rearrangements to the genome. Ultimately, the cell must execute a delicate and complex balance between NHEJ, HR and the initiation of apoptosis to limit genomic damage, mutation burden and energy drain of the organism following radiation exposure.

This review will attempt to aggregate and summarize the current research to

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Deleted: Because hnRNPs play an important role in gene transcript regulation, they are thought to be significantly involved in transcriptional arrest and in regulation of transcripts necessary for DNA repair pathways following radiation exposure. The responses of hnRNPs to IR treatment cited in the literature are diverse. These responses include, tightly controlled hnRNP protein degradation, phosphorylation and extended protein half-life. Other hnRNP responses affect gene expression regulation via transcription regulation, relocalization of protein transcript complexes to the cytoplasm, stress granule accumulation and protein-protein interactions. Abnormalities in hnRNP expression levels have been linked to cancer development and radiosensitivity. Interestingly, hnRNPs seem to play a direct role in regulating DNA repair through protein-protein interactions with critical DDR proteins. In this role, hnRNPs act as more than mRNA transcript traffickers, indeed many hnRNPs are vital to cell survival. Because of that, knockout experiments have been difficult or impossible to perform and new critical knowledge may be anticipated now after the development of siRNA technology which very few studies have completed so far (Williamson et al., 2000, Roshon and Ruley, 2005, Moumen et al., 2005). ¶ . One of the rare examples of hnRNP knock out experiments points out the role of hnRNPs in differentiation. Mouse embryonic stem cells knocked out for hnRNP C require 3-4 weeks to develop embryonic bodies with morphologically distinct cell types like blood islands and beating cardiac tissue, while wild type controls reach this stage of differentiation in 1-2 weeks (Williamson et al., 2000). . Many studies have elucidated relationships between specific hnRNPs and the formation of specific tissue types. Immunohistochemistry performed on human cervical epithelium tissue samples revealed an inverse correlation between hnRNP H and the degree of differentiation (Oberg et al., 2005). Proteomic techniques and siRNA knockdown in N1E-115 cells have shown that hnRNP K represses Cyclin-dependent kinase inhibitor 1A (p21) enabled neuronal differentiation by binding the 3' CU rich portion of p21's mRNA precursor (Yano et al., 200 ... [1]

provide a basis for further lines of inquiry. There are many examples in the literature wherein hnRNP proteins have been shown to respond to stresses which may overlap with the effects of IR including ultraviolet (UV) irradiation and various DNA damaging chemicals like cisplatin or phleomycin. Exhaustive study of these citations is the topic for a different review and such data will be used only when they corroborate evidence of IR effects on hnRNP.

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hnRNP A/B family of proteins

The hnRNP A/B family together constitutes 60% of the hnRNP present in a cell (Beyer et al., 1977). The family includes multiple proteins and splice variants, most notably hnRNP A1, A2, B1 and B2. The hnRNP A/B family shares significant structural homology, each member has two RNP-motif RNA-binding domains and a variable glycine-rich auxiliary domain at the carboxyl terminus (Dreyfuss et al., 1993). All hnRNP A/B family proteins shuttle dynamically between the nucleus and cytoplasm (Pinol-Roma and Dreyfuss, 1992). However, different proteins and splice variants have shown different expression profiles across cell types (Kamma et al., 1999) and have been implicated in a variety of tasks differentiated by protein and splice-variant.

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hnRNP A1

HnRNP A1 protein and its splice variants comprise the most common gene products in the hnRNP A/B family (Beyer et al., 1977). HnRNP A1 regulates alternative splicing of RNA polymerase II transcripts in conjunction with the serine/arginine rich protein family (Mayeda and Krainer, 1992, Caceres et al., 1994, Yang et al., 1994).

Splice variant Unwinding Protein 1 (UP1) plays a critical role along with the Telomerase Reverse Transcriptase (TERT) and the Telomerase-Associated Protein 1 (TEP1) in forming the telomerase holoenzyme responsible for telomere maintenance and promoting cell line immortalization (LaBranche et al., 1998, Fiset and Chabot, 2001, Dallaire et al., 2000, Lin et al., 2001). Several reports directly link changes in hnRNP A1 expression to the cellular response to IR (Khodarev et al., 2001, Jen and Cheung, 2003, Lin et al., 2001), others indicate responses to other DNA damage inducing agents, UV (Rundhaug et al., 2005, van der Houven van Oordt et al., 2000) and cisplatin (Yim et al., 2006). Others suggest that hnRNP A1 is affected by more general cellular stresses such as hypoxia (Denko et al., 2000), heat shock (Guil et al., 2006) and osmotic stress (Guil et al., 2006, van der Houven van Oordt et al., 2000).

Messenger RNA for hnRNP A1 was identified by gene expression microarrays to be among 126 other radiation responsive transcripts, in a screen of pooled lymphoblast isolates from 10 individuals. Isolated cells were cesium-137 (Cs-137) γ-irradiated with 3 or 10 Gy (gray) ex vivo and RNA samples were collected at 0, 1, 2, 6, 12 and 24 hours following irradiation. After 3 Gy exposure, cells' hnRNP A1 transcript expression peaked after 2 hours with expression levels 2 times the basal amount followed by a rapid decline to basal levels by the sixth hour post irradiation. One hour following 10 Gy irradiation, cells' hnRNP A1 mRNA expression increased two fold and remained high through the remaining 23 hour time period (Jen and Cheung, 2003). Khodarev et al. analyzed microarray data from U87 and HEL-C fibroblasts grown in culture treated with 0, 1, 3, or 10 Gy followed by 5 hours of incubation. Results indicate that U87 cells show a consistent decline in hnRNP A1 transcript quantity, relative to controls, after 1, 3 and

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10 Gy treatments with values of 0.99, 0.57 and 0.39 respectively. HEL-C fibroblasts demonstrated the opposite trend, increasing in transcript quantity with higher doses (1.65 fold after 1Gy, 1.81 fold after 3 Gy, and 4.53 after 10Gy exposure) (Khodarev et al., 2001).

hnRNP A1 splice variant UP1 and hTERT compose the telomerase holoenzyme. It was found that telomerase activity is downregulated over a 48 hour period following 30 Gy exposure in Jukat and CEM-6, but not Raji cells (Lin et al., 2001); Lin et al. found that hTERT was upregulated in the same period both as mRNA by Real Time PCR (RT-PCR) and protein by Western Blot. However, these authors did not explore the effects of exposure on UP1 expression directly nor hypothesized a role for the protein in the subsequent down-regulation of telomerase activity (Lin et al., 2001).

Immunofluorescence studies have revealed cytoplasmic accumulation of hnRNP A1, induced following shortwave ultraviolet (UV-C) irradiation (180 J/m^2), in 3-15% of NIH-3T3, COS, 293 and HeLa cell lines detectable 2 hours following treatment and peaking at 5 hours (van der Houven van Oordt et al., 2000). Osmotic stress in the same study resulted in similar cytoplasmic accumulation of hnRNP A1 (van der Houven van Oordt et al., 2000). The authors showed that p38 kinase activation was both necessary and sufficient to cause cytoplasmic accumulation of hnRNP A1 (van der Houven van Oordt et al., 2000). Other studies have reported stress granule accumulation of hnRNP A1, observed by immunofluorescence, in NIH-3T3 cells in response to heat shock, a high osmolarity environment and oxidative stress (Guil et al., 2006).

Given the cumulative evidence gathered, the simplest explanation for the action of hnRNP A1 is that in response to IR it accumulates in the cytoplasm and stress granules.

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This migration could play a role in targeting and silencing of bound transcripts as well as optimizing cellular resources for stress response activity. Depletion of nuclear levels of functional hnRNP A1 may contribute to a reduction of telomerase activity, progression through the cell cycle, and in some cell lines precede changes in hnRNP A1 production through unknown mechanisms in the subsequent hours or days. Given these conclusions, hnRNP A1 may be an important molecule to study to provide targeted treatment for infinitely proliferating cells and to further understand the IR response pathway.

hnRNP A2/B1

HnRNP A2 and hnRNP B1 are two closely related splice variants of the hnRNP A/B family which differ by a 12 amino acid stretch present near the amino terminus of hnRNP A2 (Burd et al., 1989). Because of this close homology, the two are often treated together in the literature under the name hnRNP A2/B1. However, important distinctions between these variants have been shown. HnRNP A2 and hnRNP B1 show different expression profiles (Kamma et al., 1999). For example, hnRNP A2 is expressed at higher levels than hnRNP B1 in the adrenal gland and brain, while B1 is more prevalent in the intestines, heart and lung in adult rats (Kamma et al., 1999). Notably, hnRNP B1 expression levels are elevated in a variety of cancers including lung (Sueoka et al., 1999, Sueoka et al., 2001, Hamasaki et al., 2001), oral cavity (Goto et al., 1999) and esophagus(Matsuyama et al., 2000), and this overexpression can be used to detect the early stages of premalignant lesions(Sueoka et al., 2001, Goto et al., 1999).

Three studies have implicated hnRNP A2/B1 induction or relocalization as an effect of irradiation or UV exposure. Gamble et al. used two-dimensional polyacrylamide gel electrophoresis to identify protein expression changes four hours following 0.5 Gy Co-60 γ-ray exposure of a human lung cell line. Among 7 downregulated proteins identified, they found hnRNP A2/B1 with a four fold lower expression following treatment compared to controls (Gamble et al., 2000). Takao et al. performed microarray analysis of freshly cultured human keratinocytes 6 hours following 100 J/m² UV-B irradiation. The hnRNP B1 mRNA transcript was identified among 88 other upregulated transcripts, and showed a 2.8 fold increase in transcript expression relative to controls (Takao et al., 2002). Cytoplasmic accumulation of hnRNP B1 was observed in 10-15% of green monkey kidney cells following UV exposure of 180 J/m² or osmotic shock as observed by immunofluorescence (van der Houven van Oordt et al., 2000).

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It has also been reported that IR can lead to DNA-protein cross links with hnRNP A2/B1 (Barker et al., 2005). HnRNP A2/B1 was identified among 29 proteins in a DNAzol-Strip screen for DNA protein crosslinks following 1 Gy Co-60 γ-ray ionizing irradiation under hypoxic conditions in Chinese Hamster Ovary cells (Barker et al., 2005). Interestingly, hnRNP A2/B1 has been proposed to inhibit DNA-PK following observations of co-immunoprecipitation of these proteins and in conjunction with a finding that rates of DSB repair increase following small interfering RNA (siRNA) knockdown of hnRNP A2/B1 in human bronchial epithelial cells (Iwanaga et al., 2005). Considering that DNA-PK is a major contributor to NHEJ, the effects of hnRNP B1 on the choice of repair mechanism, and therefore repair fidelity, may be significant (Figure

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1a). The authors suggest that hnRNP A2/B1 may play a role in cancer promotion through the erroneous repair of DSB in hnRNP B1 upregulated tissues (Iwanaga et al., 2005).

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Taken together, these results suggest an important and under-characterized role for hnRNP A2/B1 in radiation responses and cancer development specifically through the repair of DSB. Induction of hnRNP A2/B1 transcripts following radiation exposure shows some cell-type specificity and will require more exhaustive work on both A2 and B1 splice variants to resolve the question of the promotion or inhibition of pathways involved. While evidence reported by Iwanaga et al. that hnRNP B1 induction may lead to the erroneous repair of DSB offers a tantalizing proposition, this line of research is still understudied, especially as it applies to the radiation response pathway.

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A18 hnRNP (CIRP)

A18 hnRNP is a stress inducible protein, which was first cloned by low ratio hybridization subtraction of rapidly induced transcripts in UV-irradiated Chinese hamster ovary cells (Fornace et al., 1988) and then in human cell lines (Sheikh et al., 1997). A protein with 90% amino acid homology was identified as a cold-inducible RNA-binding protein (CIRP) in mice (Nishiyama et al., 1997). A18 hnRNP has one conserved RNA binding domain and several repeats of an RGG box – a single-stranded nucleic acid binding motif (Kiledjian and Dreyfuss, 1992).

A18 hnRNP has been shown to bind selectively to the 3' untranslated region of numerous mRNA sequences (Yang and Carrier, 2001). Of 46 mRNA transcripts identified to bind selectively to A18 hnRNP, about 40% are known to be stress or UV responsive (Yang and Carrier, 2001). An RKO cell line transfected with an antisense

vector to reduce hnRNP A18 expression becomes UV sensitive (Yang and Carrier, 2001). Another major group of A18 hnRNP binding mRNA's are those coding for ribosomal proteins, which also play a role in stress responses (Sheikh and Fornace Jr). Interestingly, one of the transcripts found to associate with A18 hnRNP via its 3' untranslated region is replication protein A (RPA), which plays major direct and indirect roles in DNA replication and repair, most importantly in DSB repair through HR (Fanning et al., 2006). This finding suggests a regulatory role for A18 hnRNP in the DDR pathway (Figure 2a). Moreover, RPA and A18 hnRNP are involved in the phosphorylation of tumor protein 53 (p53) (Ljungman, 2007), linking p53 with the transcriptional stress response.

In response to UV exposure, A18 hnRNP protein is induced, peaking 4 hours after treatment (Yang and Carrier, 2001, Fornace et al., 1988). After UV-induction, the protein binds to and stabilizes stress-inducible mRNA transcripts (Yang and Carrier, 2001) and translocates them from the nucleus to the cytosol (Yang and Carrier, 2001). UV treatment leads to A18 hnRNP phosphorylation of the RGG domain by glycogen synthase kinase 3 beta (GSK3β) (Kim and Kimmel, 2000), which increases its transcript binding efficiency by two-fold (Yang et al., 2006). It is suggested that this phosphorylation may also play a role in cytoplasmic relocation of A18 hnRNP because of a similar relocation pathway discovered for protein nuclear factor of activated T-cells (NFATc) (Beals et al., 1997).

In a microarray analysis of human MOLT4 cells, induction of A18 hnRNP was found immediately after 5 Gy Cs-137 γ-irradiation and lasted 8 hours (Barenco et al., 2006). In addition, one of transcripts known to selectively bind to A18 hnRNP is

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thioredoxin, which is known to be induced by X-ray irradiation, sources of oxidative stress and UV irradiation (Funasaka and Ichihashi, 1997). Thioredoxin has been shown to be upregulated in some tumors and been associated with UV resistance (Berggren et al., 1996, Grogan et al., 2000, Yokomizo et al., 1995).

A18 hnRNP protein seems to be involved in coordinating the translocation of stress-response transcripts to the cytoplasm during the recovery period following exposure to a cellular stress. The response is general, apparently encompassing stresses ranging from IR to heat-shock (Fornace et al., 1988). At the same time, protein RPA, regulated by A18 hnRNP is involved in HR DNA repair.

Other Miscellaneous hnRNP A/B Family Results

An hnRNP A/B type transcript (accession number NM_010448) was identified by microarray among 69 downregulated transcripts in isolated bone marrow cells from C57BL mice 6 hours after a whole body Co-60 γ-irradiation with 6.5 Gy. This transcript was 3 fold lower in irradiated than in control mice (Dai et al., 2006).

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Another hnRNP A/B type transcript (accession number M65028) was identified by microarray among 126 other radiation responsive transcripts, in a screen of pooled lymphoblasts from 10 individuals (Jen and Cheung, 2003). Isolated cells were Cs-137 γ-irradiated with 3 or 10 Gy and RNA was isolated at 0, 1, 2, 6, 12 and 24 hours following exposure. Protein hnRNP A/B type M65028 mRNA showed a slow decline in expression from 2 hours following either 3 or 10 Gy Cs-137 γ-irradiation and continuing throughout the 24 hour time period at the end of which its expression was 2 fold lower than control levels.

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HnRNP A3 was identified among 29 proteins in a DNAzol-Strip screen for DNA protein crosslinks following 1 Gy ionizing irradiation under hypoxic or aerated conditions in Chinese Hamster Ovary cells (Barker et al., 2005).

HnRNP C1/C2

HnRNP C1 and hnRNP C2 are splice variants which differ by a 13 amino acid stretch present in the middle of the coding sequence of the C2 gene (Burd et al., 1989). Because of their significant homology, they are frequently referred to collectively as hnRNP C1/C2 or simply hnRNP C in the literature. HnRNP C1/C2 has been shown to play a role in mRNA transcript packaging, splicing, nuclear retention and mRNA stability (Krecic and Swanson, 1999). Under normal conditions, it is located in the nucleoplasm, but not nucleoli (Lee et al., 2005). Messenger RNA and protein for hnRNP C1/C2 have been shown to respond to IR with transcript induction, chromatin-binding, chromatin-crosslinking and protein cleavage, described in studies detailed below. Because of these responses, it has been suggested that hnRNP C1/C2 plays a role in coordinating the DNA-damage response and radiation induced apoptosis pathways. However, no follow-up studies have been performed to elucidate the mechanisms of their action or the downstream effects that are dependent upon hnRNP C1/C2 responses to IR.

HnRNP C1/C2 can be isolated in screens for both DNA protein crosslinks and chromatin-binding proteins following IR. DNA crosslinking was observed one hour following treatment with moderate doses of Co-60 γ-IR (1-4 Gy) in Chinese hamster ovary cells (CHO AA8) and human fibroblast cells (GM00637) especially under hypoxic conditions (Barker et al., 2005). Chromatin-binding was observed in HeLa cells 3 hours

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after treatment with high doses of IR (25 Gy) (Lee et al., 2005). Such binding was found in nuclei extracts from treated cells and was reversible with 0.5 M but not 0.35 M NaCl elution (Lee et al., 2005). These binding and crosslinking responses imply close proximity between hnRNP C1/C2 and chromatin. However, the localization pattern of hnRNP C1/C2's does not change after exposure to ionizing irradiation (Lee et al., 2005, van der Houven van Oordt et al., 2000), therefore hnRNP C1/C2 does not appear to bind specifically to damage sites. The authors suggest that hnRNP C1/C2 may play a 'global' role in orchestrating DNA repair pathways. It is possible that hnRNP C1/C2 coordinates changes in gene expression required for DNA repair pathways after irradiation through direct interaction with genomic DNA, DNA associated proteins and/or mRNA transcripts, but further study is required to validate this hypothesis.

A different report has indicated that hnRNP C1/C2 undergoes cleavage as the result of activation of the apoptotic proteases at 8 hours post treatment by high doses of IR (20 Gy) in Burkitt's lymphoma cells, BL30A (Waterhouse et al.). Importantly, this response was not observed in a radiation resistant subline, BL30K, but could be induced in BL30K by a variety of apoptotic agents (etoposide, C8 ceramide, tetrandrine). The cleavage seems to be one result of a more general apoptosis pathway (Waterhouse et al.). No follow-up studies were done to elucidate further the mechanisms or downstream effects of this cleavage.

Two microarray studies have indicated a change in expression levels of hnRNP C1/C2 transcripts following IR treatment. One study reported steadily decreasing transcript levels of hnRNP C1/C2 in the 24 hour period following 3 Gy, but not 10 Gy Cs-137 γ-irradiation treatment of 10 different human lymphoblastoid cell lines (Jen and

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Cheung, 2003). Another study found a 5x decrease in transcript expression relative to control levels 6 hours after 6.5 Gy Co-60 γ-irradiation treatment of bone marrow cells extracted from C57BL mice as compared to a sham irradiated control (Dai et al., 2006). While these results imply a change in the transcription of hnRNP C1/C2 following radiation exposure, once again no follow-up studies were performed to connect these changes in expression levels to downstream effects on cells.

Experiments examining general stress response pathways have implicated a role for hnRNP C1/C2 in DNA damage repair mechanisms. One study showed that hnRNP C1/C2 binds to Ku protein if it is at the same time binding RNA transcripts and can be phosphorylated by the catalytic subunit of the DNA-PK complex (Figure 1b) (Zhang et al., 2004). This suggests a possible role for hnRNP C1/C2 in DNA DSB repair through the non-homologous end-joining pathway (Lee et al., 2005). Other studies have connected hnRNP C1/C2 with telomere repair and maintenance. It has been shown using UV cross-linking and co-immunoprecipitation assays that hnRNP C1/C2 associates with the 6 bp U-tract of the RNA component of the human telomerase holoenzymes in lung adenocarcinoma cells (H1299) (Ford et al., 2000). Telomerase activity assays on VA13 cells revealed that the association of hnRNP C1/C2 with telomerase correlates with its ability to access the telomere (Ford et al., 2000).

It is intriguing to speculate that the previously mentioned responses of hnRNP C1/C2 to IR are responsible for regulation of DDR pathways induced by IR and general maintenance of genomic stability. It may be that during stress-induced transcriptional arrest hnRNP C1/C2 lacks bound RNA and therefore stops interacting with Ku and

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DNA-PK, perhaps leading to changes in NHEJ activity. Further study is required to prove these hypotheses.

HnRNP K

HnRNP K has been implicated in the processes of chromatin remodeling and mRNA transcription, splicing, export and translation (Bomsztyk et al., 1997). It shuttles between the nucleus and cytoplasm and contains three RNA-binding domains which preferentially bind C-rich nucleic acids and protein interactive domain (Klimek-Tomczak et al., 2004). This section will focus on work performed by the Moumen lab showing that hnRNP K acts as a transcriptional co-regulator of p53 targets following irradiation (Figure 3).

While p53's role in cell-cycle checkpoint arrest and the DDR pathway was identified 15 years ago by Lane et al (Lane, 1992) (Efeyan and Serrano, 2007), the work of the Moumen group on hnRNP K was not carried out until 2003. It is not surprising that an important regulatory protein like p53 has a corresponding transcript regulating hnRNP partner. The extensive work done by the Moumen group to elucidate the function of hnRNP K in this role has provided an example for future lines of research and added a new critical molecule to incorporate into models of DNA damage repair and other cellular responses to IR.

Proteomic analysis first identified hnRNP K among upregulated proteins in GM 14680 cells treated with 20 Gy of γ-IR. Subsequent immunoblot experiments revealed that hnRNP K protein levels increased 2-3 fold 15 minutes after treatment with IR or the radiomimetic drug phleomycin (Moumen et al., 2005). These protein levels returned to

control level after 3 hours in repair-proficient cells, but not in a line of cells deficient in DSB repair (180BR) in which hnRNP K expression remained elevated 12 hours following irradiation (Moumen et al., 2005).

Through siRNA knockdown experiments in SAOS2 cells Moumen et al. found that hnRNP K protein, like p53, is induced following irradiation due to cessation of the human homologue of murine double minute (HDM2) regulated ubiquitin based proteasomal degradation (Moumen et al., 2005). They went on to show, through further chemical inhibition and siRNA knockdown experiments that, like p53, hnRNP K induction was dependent on the action of ATM and ATR kinases (Figure 3b). ATM and ATR kinases are key regulatory proteins in the DDR pathway and function to phosphorylate and inhibit degradation of DDR proteins following IR (Abraham, 2004, Shiloh et al., 2004).

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To test for the specificity of observed hnRNP K induction, the group measured the effects of UV, heat shock, hypotonic and hypertonic treatments on hnRNP K protein expression in multiple cell lines. Of these, only UV (25 J/m^2 measured one hour after treatment) lead to hnRNP K upregulation (Moumen et al., 2005). The author's suggest that the induction pattern of hnRNP K indicates that it is upregulated specifically in response to DNA lesions (Moumen et al., 2005)

Interestingly, Chromatin Immunoprecipitation (CHiP) assays indicated that hnRNP K and p53 are co-recruited to p53 responsive promoters and that hnRNP K acts to regulate mRNA targets of p53 controlled genes (Figure 3c) (Moumen et al., 2005). Cells treated with siRNA targeting hnRNP K prevented p53 recruitment to cyclin-dependent kinase inhibitor 1A (p21) and HDM2 promoter regions despite the fact that p53

stabilization was not affected (Moumen et al., 2005). Further, RT-PCR analysis of the U2OS cell line assessing expression of p53 transcript targets: p21, HDM2, 14-3-3σ (stratifin) and luciferase, confirmed that hnRNP K knockdown inhibited induction of these mRNA transcripts following irradiation (Figure 3d) (Moumen et al., 2005).

Because p53 plays a critical role in cell cycle arrest following an IR event, the authors went on to explore the role that hnRNP K might play in facilitating this p53-dependent cell cycle arrest. After hnRNP K knockdown by siRNA, γ-irradiated MRC5 fibroblast cells did not show their natural G1/S phase arrest (Moumen et al., 2005). Similarly, U2OS cells which are normally arrested at the G2/M phase following irradiation, did not undergo cycle arrest when hnRNP K or p53 siRNA treatment was applied (Moumen et al., 2005). However, p53-deficient SAOS2 cells exhibited their characteristic G2/M phase arrest following irradiation regardless of hnRNP K knockdown (Moumen et al., 2005). These results suggest that hnRNP K is an essential cofactor of p53 dependent cell cycle checkpoints following IR.

From the Moumen lab's report, it is clear that hnRNP K is in part responsible for the fate of p53 regulated transcripts following irradiation. HnRNP K is one of downstream targets of ATR/ATM phosphorylation and HDM2 mediated degradation pathways (Figure 3b,e). In this, hnRNP K mirrors the behavior of p53 and ultimately acts as a co-factor coordinating the downstream response of DDR pathways. However, the mechanisms and regulatory steps occurring that deliver hnRNP K-target transcripts to translation sites in the cytoplasm have yet to be elucidated. Transcripts may or may not be escorted directly by hnRNP K to the cytoplasm. Irrespective of that, these transcripts

are almost certainly interacting with a variety of other regulatory proteins and RNA complexes before their ultimate translation.

Given hnRNP K's relationship to p53 and its transcript targets, it is not surprising that hnRNP K overexpression has been linked to multiple forms of cancer (Pino et al., 2003). Both yet-to-be-discovered transcript shuttling pathways and hnRNP K itself are prospective targets for anticancer therapies.

hnRNP P2 (TLS/FUS)

Protein hnRNP P2 more commonly known as TLS, FUS or TLS/FUS (Calvio et al., 1995) contains an RNA binding motif flanked by RGG repeats (Delattre et al., 1992, Crozat et al., 1993, Rabbitts et al., 1993). In some human sarcomas and leukemias, the C-terminus of this protein is missing and the remaining amino acid sequence is fused to sequences coding unrelated transcription factors (Kuroda et al., 2000). These fusion products are invariably associated with myxoid and round cell liposarcomas (Crozat et al., 1993, Rabbitts et al., 1993).

Transgenic hnRNP $P2^{-/-}$ mice studied by Kuroda et al. display increased radiosensitivity, profound defects in spermatogenesis, and a mild defect in somatic growth as compared to wild type mice.(Kuroda et al., 2000) In a survival study 18/20 adult transgenic mice died within 20 days of receiving whole body Co-60 γ-rays irradiation with 7 Gy, whereas control mice survived in 21/26 cases. Additionally, primary mouse embryonic fibroblasts isolated from hnRNP $P2^{-/-}$ mice showed consistently lower survival rates than wildtype isolated in the 7 days following 3.5, 7, or 10.5 Gy irradiation (Kuroda et al., 2000).

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The authors have proposed that hnRNP P2 may play a role in homologous DNA pairing and recombination, key aspects of HR repair (Figure 2b). With the evidence gathered thus far, there can be little doubt that hnRNP P2 plays a role in maintaining genomic stability, especially following genotoxic stresses. The cancer associated fusion proteins are likely to play a critical role in facilitating structural changes to the genome necessary for the development of malignancy. However, more work is needed to elucidate the mechanistic underpinnings of hnRNP P2's role in genomic maintenance.

Miscellaneous Results

During the course of research a number of references were collected that showed a link between hnRNP transcript induction and IR, but did not fit with a greater body of evidence. These sources have been collected and summarized in (Table I). It is important to note that these citations are biased towards positive results and the majority of microarray or proteomic studies involving IR treatment in the literature do not report changes to hnRNP transcripts or proteins. For this reason it may be assumed that the results accumulated are relevant to the particular treatment conditions of each experiment and not necessarily generalizable.

Even with the above qualifications, there are some interesting conclusions to be drawn from the accumulated gene expression results. Some hnRNP are more represented than others when associated with gene expression changes following IR. Namely hnRNP E gene family members were each differentially regulated in three IR studies; hnRNP H, R and U were each noted in two studies; and hnRNP D, L and M were noted in only one

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study each. These results should guide the course of mechanistic research towards promising candidate hnRNP that are involved in DDR following irradiation.

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Discussion

From the evidence accumulated, it is clear that hnRNP are critical regulatory proteins in the cellular response to IR and other stresses. It is also clear from the diversity of responses that have been noted, that biology is only beginning to characterize the role of these multi-faceted proteins in DDR regulation. Advances in molecular biology techniques, especially siRNA and florescence assays, will prove to be critical in furthering our understanding.

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The most commonly observed effect of IR on hnRNP was transcript or protein induction. Sixteen hnRNP, A1, A2/B1, A18, AB Type (NM_010448), AB Type (M65028), C1/C2, D, E1, E2, H1, H3, K, L, M, R and U were noted in various studies as being IR inducible mRNA. Most of the observed effects appear to be cell type or growth phase specific and it is difficult to draw broad conclusions from the data. Rather, the induction effects thus far noted may serve as fruitful jumping off points for future research, especially in the cases of hnRNP E, H, R and U which have each been noted in multiple induction studies following IR, but lack followup research to describe the relevant DDR pathways they might connect to.

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While hnRNP are characterized primarily as RNA transcript processing agents, there is a surprising lack of evidence of hnRNP relocalization to the cytoplasm or stress granules following IR treatment. HnRNP A1 and B1 relocalization has been noted in response to a variety of stresses, including UV, but not to IR treatment specifically (van

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der Houven van Oordt et al., 2000, Guil et al., 2006). It is likely that the dearth of evidence directly linking IR treatment and hnRNP relocalization is due to a lack of investigation or the technical difficulties associated with IR studies. Further investigations into this response are likely to be fruitful, especially in the examples of hnRNP A1 and B1.

The most important evidence to emerge from the literature reviewed in this paper demonstrates that hnRNP function as upstream and co-dependent regulators of critical DDR proteins. Based on the data found in the literature thus far, it is possible to hypothesize that hnRNP optimize the cell's DDR response by regulating proteins which participate in HR, NHEJ and general transcriptional arrest.

HnRNP K has been shown to act as a critical co-factor of p53, necessary for p53-dependent cell cycle checkpoints and p53-dependent transcript induction following IR treatment. Further, like p53, hnRNP K is a downstream target of ATR/ATM phosphorylation and HDM2 mediated degradation which coordinates its rapid induction following irradiation (Moumen et al., 2005). Also, stress induced cytoplasmic relocalization of hnRNP A1 corresponds to a reduction in telomerase activity (of which hnRNP A1 is a constituent) which contributes to cell cycle arrest following IR (Khodarev et al., 2001). Connection between hnRNP C1/C2 and telomerase activity were also noted and may be involved in cell cycle arrest.

Other hnRNP have been closely associated with HR and NHEJ DSB repair pathways. UV and IR have been shown to produce positive or negative induction of hnRNP B1 (Gamble et al., 2000, Takao et al., 2002). Upregulation of hnRNP B1 leads to DNA-PK inhibition (Iwanaga et al., 2005) and would favor the HR pathway, while

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downregulation of hnRNP B1 would favor NHEJ. Many general stresses have been shown to upregulate A18 hnRNP, including UV and IR treatment, which in turn upregulates RPA favoring HR repair (Fornace et al., 1988, Yang and Carrier, 2001, Fanning et al., 2006, Barenco et al., 2006). The interaction of hnRNP C1/C2 with Ku and DNA-PK is dependent on an interaction between hnRNP C1/C2 and mRNA (Zhang et al., 2004), which are generally downregulated in response to stress because of transcriptional arrest. The dissociation of hnRNP C1/C2 with Ku and DNA-PK almost certainly has implications in the rate of NHEJ, which DNA-PK and Ku regulate, however it is difficult to determine if this would favor or oppose NHEJ without further evidence. Lastly, hnRNP P2 has been shown to be directly involved in HR repair and hnRNP $P2^{-/-}$ mice are radiosensitive (Calvio et al., 1995).

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Conclusions

In light of the above associations between hnRNP and critical components of DDR, it is not surprising that mutations in a number of these proteins, hnRNP A18, B1, K, P2 and L, have been linked to cancer and radiosensitivity (Yang and Carrier, 2001, Sueoka et al., 1999, Sueoka et al., 2001, Hamasaki et al., 2001, Goto et al., 1999, Matsuyama et al., 2000, Pino et al., 2003, Kuroda et al., 2000, Edick et al., 2005). It seems that hnRNP proteins are integral in determining the delicate balance of mutation prone and high fidelity repair processes, prevention of genomic rearrangements, orchestration of transcript mobilization post IR stress, and cooperation with transcription factors such as p53. For these reasons, the continued study and elucidation of hnRNP

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holds the promise of better understanding the mechanisms which promote cancer and ultimately may lead to useful treatments.

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Figure Legends:

Figure 1: Non-homologous End Joining Repair (NHEJ)

NHEJ repairs DSB through an error prone mechanism available at any point during the cell cycle. As opposed to HR, NHEJ is less likely to lead to gross chromosomal rearrangements. a.) UV and IR are known to induce hnRNP A2/B1 protein which has been proposed to inhibit DNA-PK (Iwanaga et al., 2005). b.) hnRNP C is phosphorylated by a complex of DNA-PK and Ku proteins when it is bound to RNA transcript; the effect on NHEJ is unknown (Lee et al., 2005, Zhang et al., 2004). c.) The canonical NHEJ pathway is shown repairing a DSB beginning with DNA-PK and Ku protein binding, followed by Rad50, Nbs1, and Mre11 recruitment, displaced by XRCC4 which recruits DNA-Ligase IV culminating in the rejoining of the broken DNA strands (Weller et al., 2004)(Sonoda et al., 2006, Weinstock et al., 2006, Weller et al., 2004).

Figure 2: Homologous Recombination (HR)

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HR repairs DSB using the broken strand's sister chromatid as a template. As opposed to NHEJ, HR may restore the complete sequence information for damaged genes. However, an excess of HR may lead to gross chromosomal rearrangements. **a.)** Many forms of stress including UV and IR are known to induce A18 hnRNP which exports RPA transcripts to the cytoplasm (Yang and Carrier, 2001). Cytoplasmic localization likely induces RPA which would facilitate HR. **b.)** Kuroda's group has found evidence that hnRNP P2 promotes homologous repair in mice through an unknown mechanism. The authors suggest that hnRNP P2 may provide an RNA template for HR (Kuroda et al., 2000). **c.)** A simplified view of the HR pathway details the roles of Rad51 and RPA in preparing the damaged DNA for restoration using the sister chromatid as a template (San Filippo et al., 2008).

Fig 3 hnRNP K and p53 in the DNA Damage Response (DDR)

The Moumen Lab showed that hnRNP K and p53 act as co-regulators. This figure presents a simplified view of p53's role in the DNA damage response pathway (Bullock and Fersht, 2001) alongside hnRNP K functions discovered by the Moumen lab (Moumen et al., 2005) . **a.)** Following DNA damage due to IR or other insult, **b.)** proteins ATM and ATR induce DNA PK and CKII which phosphorylate p53 and hnRNP K. **c.)** Phosphorylated hnRNP K and p53 proteins bind co-dependently to p53 promoter sites resulting in transcript upregulation for a number of DDR genes. **d.)** P53/hnRNP K targets, p21 and 14-3-3σ halt cell cycle progression at G1 and G2 phases respectively. **e.)** Another p53/hnRNP K target, MDM 2 plays an inhibitory role by adding ubiquitin to p53 and hnRNP K resulting in their degradation.

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Because hnRNPs play an important role in gene transcript regulation, they are thought to be significantly involved in transcriptional arrest and in regulation of transcripts necessary for DNA repair pathways following radiation exposure. The responses of hnRNPs to IR treatment cited in the literature are diverse. These responses include, tightly controlled hnRNP protein degradation, phosphorylation and extended protein half-life. Other hnRNP responses affect gene expression regulation via transcription regulation, relocalization of protein transcript complexes to the cytoplasm, stress granule accumulation and protein-protein interactions. Abnormalities in hnRNP expression levels have been linked to cancer development and radiosensitivity. Interestingly, hnRNPs seem to play a direct role in regulating DNA repair through protein-protein interactions with critical DDR proteins. In this role, hnRNPs act as more than mRNA transcript traffickers, indeed many hnRNPs are vital to cell survival. Because of that, knockout experiments have been difficult or impossible to perform and new critical knowledge may be anticipated now after the development of siRNA technology which very few studies have completed so far (Williamson et al., 2000, Roshon and Ruley, 2005, Moumen et al., 2005).

One of the rare examples of hnRNP knock out experiments points out the role of hnRNPs in differentiation. Mouse embryonic stem cells knocked out for hnRNP C require 3-4 weeks to develop embryonic bodies with morphologically distinct cell types like blood islands and beating cardiac tissue, while wild type controls reach this stage of differentiation in 1-2 weeks (Williamson et al., 2000).

Many studies have elucidated relationships between specific hnRNPs and the formation of specific tissue types. Immunohistochemistry performed on human cervical

epithelium tissue samples revealed an inverse correlation between hnRNP H and the degree of differentiation (Oberg et al., 2005). Proteomic techniques and siRNA knockdown in N1E-115 cells have shown that hnRNP K represses Cyclin-dependent kinase inhibitor 1A (p21) enabled neuronal differentiation by binding the 3' CU rich portion of p21's mRNA precursor (Yano et al., 2005). Western blots and immunofluorescence performed on cultured rat brain sections showed that HnRNP A2 concentration in oligodendrocytes increases, especially in the extranuclear component, prior to their differentiation into MBP-positive cells (Maggipinto et al., 2004). As discussed later, elevated hnRNP A2/B1 protein levels often precede morphological differentiation in lung cancer (Sueoka et al., 2001, Goto et al., 1999). Similarly, hnRNP I (or PTB) is typically upregulated in ovarian tumors (He et al., 2007). Other studies have reported a connection between hnRNP proteins and terminal erythrocyte any erythrocyte differentiation (Ostareck et al., 1997) (Sella et al., 1999, Perrotti et al., 2002).

Finally, different tissues express hnRNP proteins in different quantities and as different splicing variants. For example, purification of hnRNP E1/E2 and K from the protein extracts of different mouse tissues by a poly-C single stranded DNA oligonucleotide column results in different hnRNP Western blot patterns (Figure 1.).

Therefore, HnRNP proteins play an important role in differentiation and development because they execute translational regulation and govern cell type specific expression (Kamma et al., 1995). Exact involvement of hnRNPs in senescence and differentiation is difficult to asses at this moment. One possible explanation is sumoylation, small ubiquitin-related modifier modification (SUMO), of hnRNPs such as hnRNP A1, F, K and L (Li et al., 2004, Rosas-Acosta et al., 2005) and the induction of

senescence in SUMO overexpressing cell lines (Li et al., 2006). In non-modified cell lines such as rat pheochromocytoma line PC12, differentiation induction by Nerve Growth Factor (NGF) leads to changes in hnRNP C1/C2, E1/E2, K and L protein quantities (Figure 2).

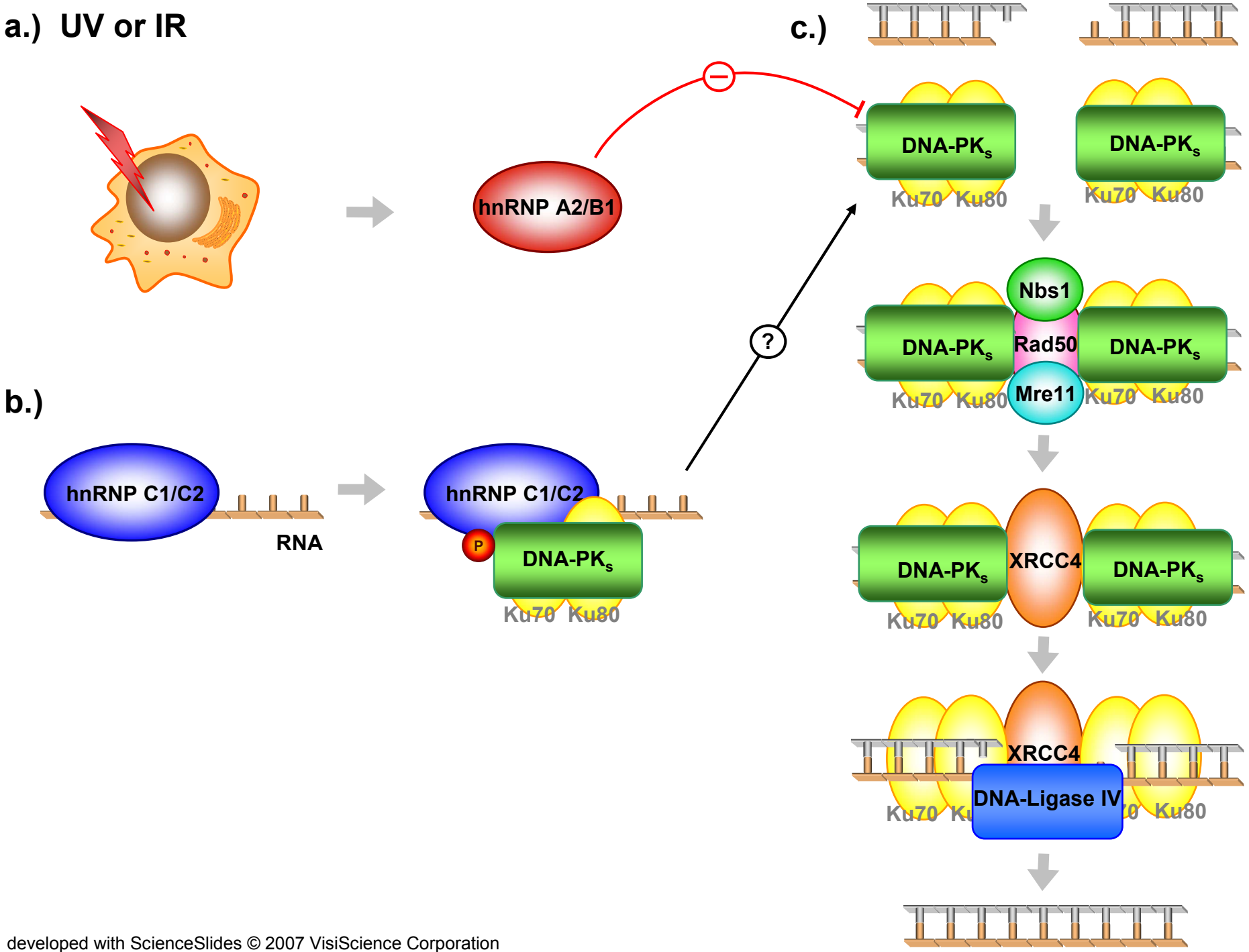

Figure 1: Non-homologous End Joining Repair (NHEJ)

NHEJ repairs DSB through an error prone mechanism available at any point during the cell cycle. As opposed to HR, NHEJ is less likely to lead to gross chromosomal rearrangements. **a.)** UV and IR are known to induce hnRNP A2/B1 protein which has been proposed to inhibit DNA-PK (Iwanaga et al., 2005). **b.)** hnRNP C is phosphorylated by a complex of DNA-PK and Ku proteins when it is bound to RNA transcript; the effect on NHEJ is unknown (Lee et al., 2005, Zhang et al., 2004). **c.)** The canonical NHEJ pathway is shown repairing a DSB beginning with DNA-PK and Ku protein binding, followed by Rad50, Nbs1, and Mre11 recruitment, displaced by XRCC4 which recruits DNA-Ligase IV culminating in the rejoining of the broken DNA strands (Sonoda et al., 2006, Weinstock et al., 2006, Weller et al., 2004).

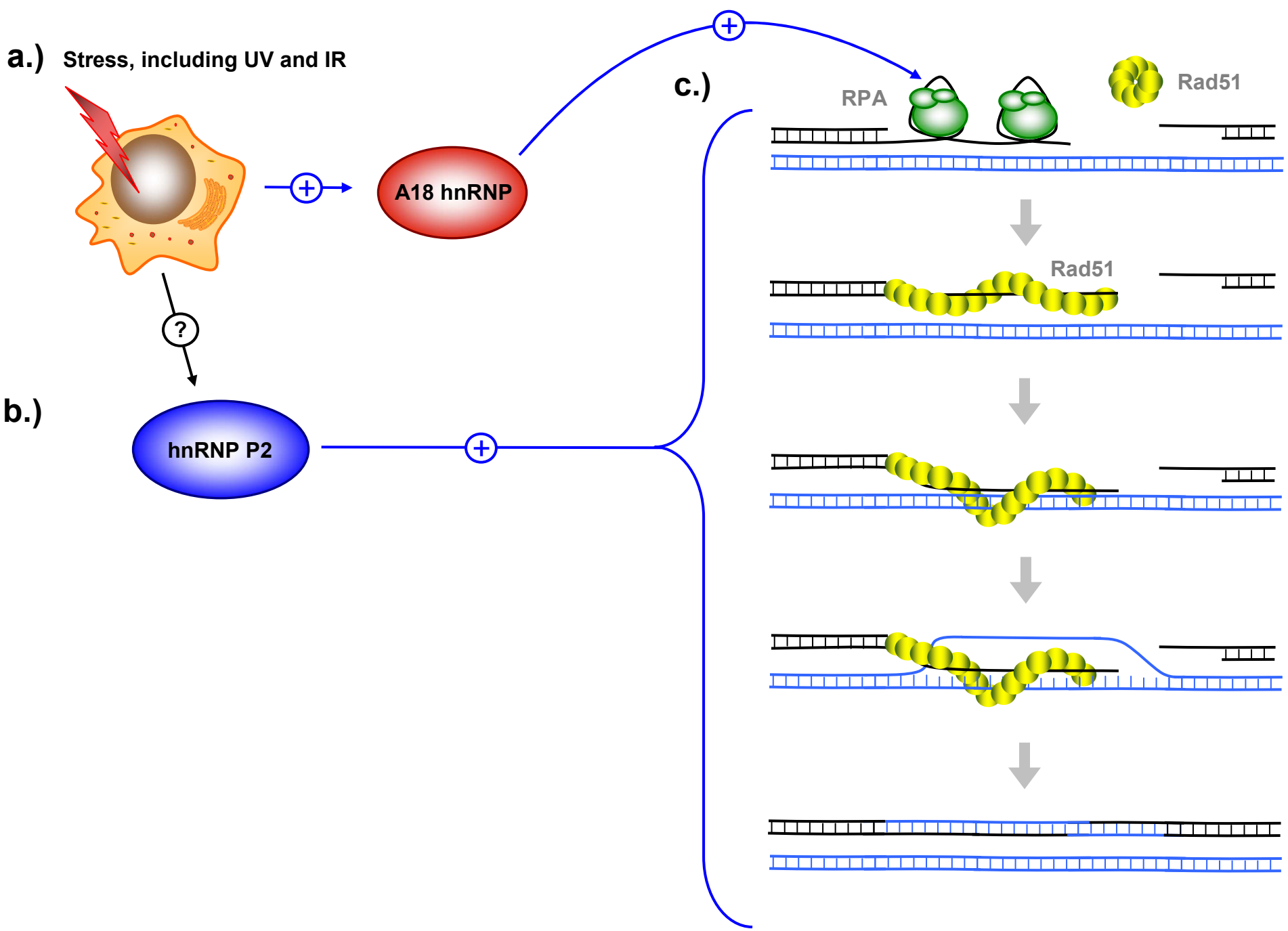

Figure 2: Homologous Recombination (HR)

HR repairs DSB using the broken strand's sister chromatid as a template. As opposed to NHEJ, HR may restore the complete sequence information for damaged genes. However, an excess of HR may lead to gross chromosomal rearrangements. **a.)** Many forms of stress including UV and IR are known to induce A18 hnRNP which exports RPA transcripts to the cytoplasm (Yang and Carrier, 2001). Cytoplasmic localization likely induces RPA which would facilitate HR. **b.)** Kuroda's group has found evidence that hnRNP P2 promotes homologous repair in mice through an unknown mechanism. The authors suggest that hnRNP P2 may provide an RNA template for HR (Kuroda et al., 2000). **c.)** A simplified view of the HR pathway details the roles of Rad51 and RPA in preparing the damaged DNA for restoration using the sister chromatid as a template (San Filippo et al, 2008).

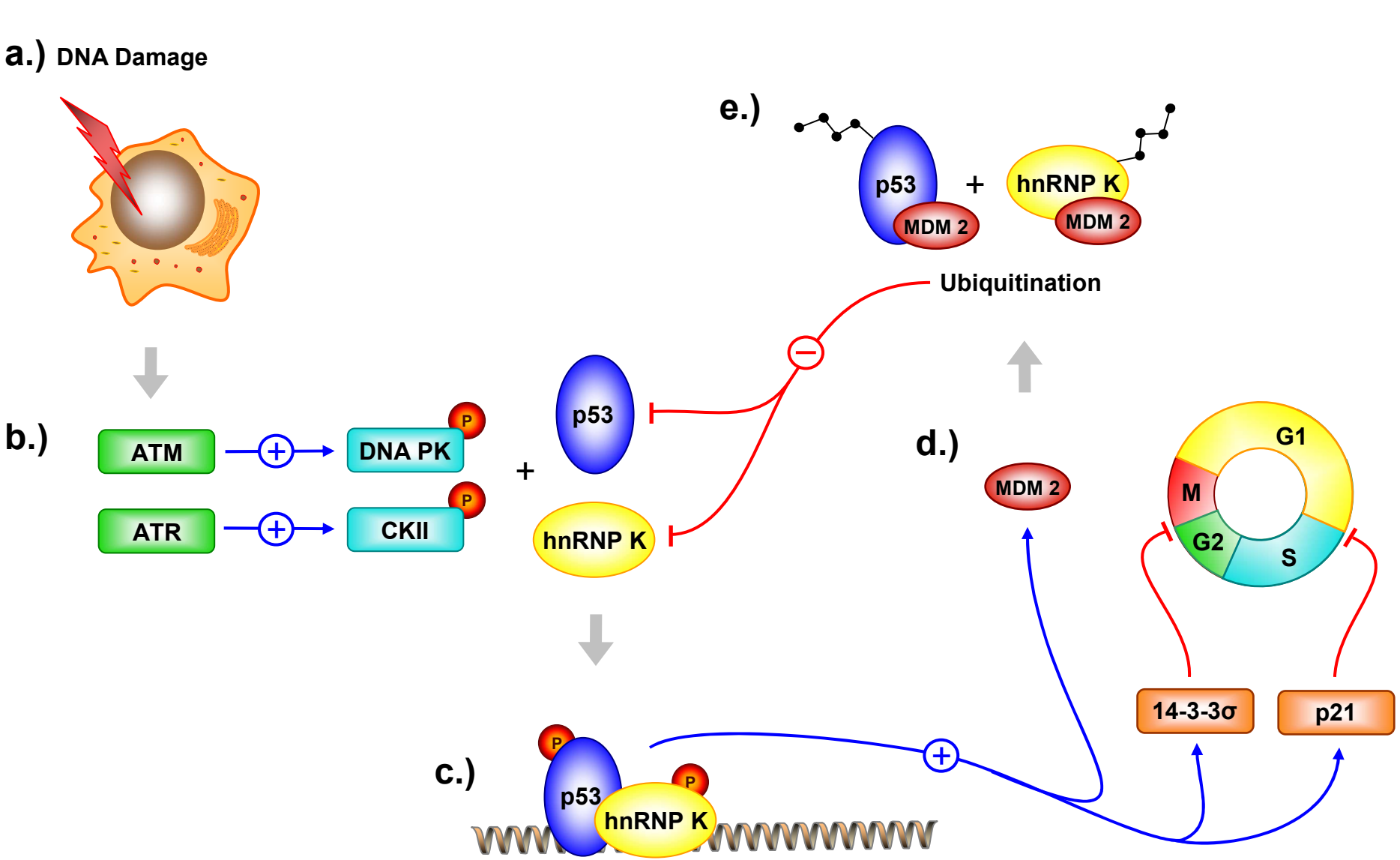

Fig 3 hnRNP K and p53 in the DNA Damage Response (DDR)

The Moumen Lab showed that hnRNP K and p53 act as co-regulators. This figure presents a simplified view of p53's role in the DNA damage response pathway (Bullock and Fersht, 2001) alongside hnRNP K functions discovered by the Moumen lab (Moumen et al., 2005) . **a.)** Following DNA damage due to IR or other insult, **b.)** proteins ATM and ATR induce DNA PK and CKII which phosphorylate p53 and hnRNP K. **c.)** Phosphorylated hnRNP K and p53 proteins bind co-dependently to p53 promoter sites resulting in transcript upregulation for a number of DDR genes. **d.)** P53/hnRNP K targets, p21 and 14-3-3σ halt cell cycle progression at G1 and G2 phases respectively. **e.)** Another p53/hnRNP K target, MDM 2 plays an inhibitory role by adding ubiquitin to p53 and hnRNP K resulting in their degradation.